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Population proteomics as a novel tool for breed characterization: practical considerations

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ABSTRACT

The aim of this study was to describe the pros and cons of a population proteomic approach aiming to characterize local chicken breeds. The experiment involved a total of 29 males of Pépoi, Padovana, and Ermellinata di Rovigo Italian local chicken breeds. Sarcoplasmic protein fractions of breast muscle were analysed by two-dimensional electrophoresis. Image analysis followed by statistical analysis enabled to differentiate groups of individuals on the basis of similarities of protein expression. Individuals well clustered into groups corresponding to the breed of origin. The Significance Analysis of Microarray analysis enabled identification of the most relevant spots regarding breed differentiation; 10 of these were identified by Mass Spectrometry, revealing preliminary evidences on the mechanisms of the breed differentiation process. The approach succeed in differentiating the individuals in groups corresponding to the different breeds, unfolding the relations among breeds and single individuals, analyzing and measuring the genetic variation at encoding loci. Results evidenced the ability to proteomic analyses to identify and to characterize chicken breeds.
(Keywords: population proteomics, characterization, chicken, local breeds)

INTRODUCTION

Proteomics is a well established technique for the separation and identification of proteins in complex samples. Although it has been used in the past for phylogenetic studies using bood or single protein polymorphisms (*Okabayashi et al.,* 1998; *Inafuku et al.,* 1998), only in recent years comparative proteomics techniques, in particular bi-dimensional electrophoresis, enabled the large-scale screening for hundreds proteins in a single step. It shows great potential in provinding highly valuable information in phylogenetic analysis, and has the ability to reveal new perspectives and lines of research (*Biron et al.*, 2006). Beyond that, proteomics complements and extends study of genomic and transcript data, reflecting true biochemical outcome of genetic information (*Doherty et al.*, 2007). Currently, just few authors used proteomics to investigate natural variation within species populations (*Biron et al.*, 2006), and despite the advances made in this discipline, there is a lack of algorithms and statistical tools for handling the impressive amount of information obtainable from such techniques (*Navas and Albar,* 2004). The aim of this study is to describe the pros and cons of a population proteomic approach aiming to characterize local chicken breeds.

MATERIALS AND METHODS

In the Veneto region of Italy the local breeds of chicken, which are typically reared in free range systems, provide an interesting alternative to commercial lines. The trial made

use of day-old chicks of three Italian local chicken breeds: Padovana (PD), Pépoi (PP) and Ermellinata di Rovigo (ER). A total of 29 males (PD=10, PP=10, and ER=9) were slaughtered at 190 d of age. At hatch, chicks were placed together in an indoor pen with access to a grass paddock. Rearing, feeding, slaughtering conditions and veterinary treatments were the same for all animals. About 15 min post mortem, 5 grams samples of muscle (*Pectoralis superficialis*) were collected from the left breast and frozen in liquid Nitrogen for the analysis.

The extraction of sarcoplasmic proteins was performed using the procedure described by *Rathgeber et al.* (1999). This protein fraction represent about 30–35% of the muscular proteins. Despite the great diversity of this class of proteins, they share common characteristics such as a relatively low molecular weight, a relatively high isoelectric point and globular structure. A total of 58 samples were analysed by two-dimensional electrophoresis (2 repetitions per animal). Protein concentration was quantified using the Bradford assay (Bio-Rad). The isoelectric focusing (IEF) was carried out using a Protean IEF cell (Bio-Rad Laboratories Inc., Hercules, CA), loading 300 μg of protein each strip (17 cm, pH 4–7 linear). SDS–PAGE was performed in a Protean XL cell (Bio-Rad Laboratories) on 12% polyacrylamide gels (2.6% bisacrylamide) at 35 mA/gel at 8 °C, until the dye track reached the end of the gels. Gels were silver stained following the protocol of *Shevchenko et al.* (1996). Gels images were acquired through a GS-800 densitometer and analysed with a computerized image analysis: Image Master 2D Platinum (GE Healthcare). Spots were excised from gels, destained and digested at 37 °C for 5 h. Details about the used protocols are available upon request. A Voyager DE-Pro model MALDI-TOF mass spectrometer (PerSeptive BioSystems) was used in positive-ion reflector mode for PMF. External calibration was performed with a standard peptide solution (Proteomix). PMFs were compared to Aves nrNCBI (12/2008, 102 448 seq) protein sequence databases using MASCOT 2.2 software. The initial search parameters allowed a single trypsin missed cleavage, partial carbamidomethylation of cystein, partial oxidation of methionine and mass deviation lower than 25 ppm.

All spots detected were included for the statistical analysis. Cluster analysis was performed using the PROC CLUSTER of SAS (1997) and the Ward's minimum variance option. Dendrograms were plotted using PROC TREE procedure of SAS. The statistical differences in protein expression among groups were tested using the Significance Analysis of Microarrays (SAM) method as described by *Meunier et al*. (2005). Spots with a Fold Change greater than 2 were retained and considered for the identification.

RESULTS AND DISCUSSION

Image analysis detected 246, 275, and 226 different spots for the PP, PD and ER breeds, respectively, respectively, showing that PD has the highest proteomic richness. For each spot, expression results were averaged to obtain a single value within individual.

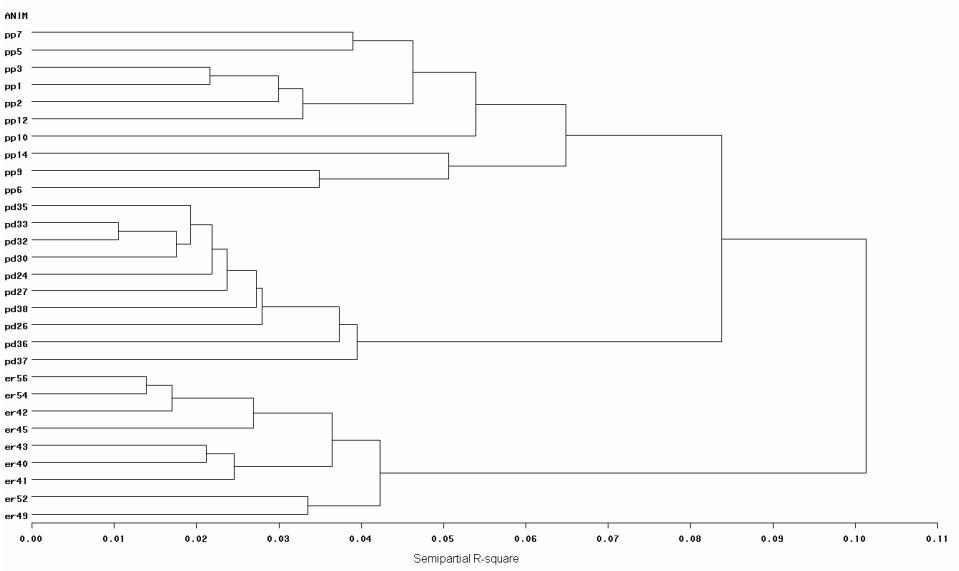
The cluster plot for PP, ER and PD is presented in *Figure 1*. Individual results always well divided into two groups, corresponding to the breeds analysed. Within each sub-cluster, individuals are differently grouped based on similarity on protein expression. The Significance Analysis of Microarrays (SAM) method was adopted to discriminate, among all "statistically" significant spots, those witch retain a "biological" significance. This was performed choosing only the spots presenting a volume ratio greater than a predefined Fold Change level. This method was studied to minimize false

positive and to avoid losing information with false negative, especially when few replicates are available. SAM analysis detected 47 differentially expressed significant spots for the confrontations among the breeds, 10 were identified by mass spectrometry. Identified proteins can be divided in two categories: breed specific spots, i.e. spots that are expressed only in a particular breed, and spots that are declared up or down expressed respect to a predefined Fold Change level (fixed to a value of 2). Identified proteins appear heterogeneous in their function. Enzymes, transport, contractile and motile, regulatory and scaffold proteins have been identified and seem hence to play a function in breed differentiation. In particular, Pépoi breed showed two up expressed proteins: GLO1 and HSPB1. GLO1 is a 184 aa long protein of the glyoxalase I family and resulted up expressed in this breed if compared to Ermellinata di Rovigo one. HSPB1, a protein involved in stress resistance and actin organization, although expressed by all analysed breeds, was up-expressed in the Pépoi (FC=4). This result could help in explaining the marked aptitude to environmental adaptation and stress resistance or at least being used to further investigate such characteristic. The Ermellinata di Rovigo breed evidenced two specifically expressed proteins: BRD4 and PGP. They are enzymes respectively involved in the process of cellular mitosis and carbohydrates metabolism, and were no detected in the other analysed breeds. The peculiar expression levels of these proteins could contribute in explaining the differences in terms of growth rates shown by this breed respect to the others. Lastly, the Padovana evidenced two breed specific proteins, CFL2 and ANXA5, and an up expressed protein, APOA1, if compared to both Ermellinata and Pépoi. CFL2 controls actin polymerization and depolymerisation, ANXA 5 is a collagen-binding protein belonging to the annexin family, while APOA1 is a protein participating to the transport of cholesterol from the tissues to the liver.

In general the approach used gave promising results. The method can successfully differentiate the individuals in groups corresponding to the different breeds. It is therefore able to analyze and measure the genetic variation at encoding loci, in a different way to the transcriptomics techniques, which, analyzing the mRNA, are not able to entirely fix the attention on the expressed genes. On the contrary to molecular markers such as SNPs or microsatellites, here the real differences among breeds, due to the different adaptation pressure and selection forces, are outlined. The possibility to isolate and identify the proteins that play the major role in the breed differentiation processes gives the chance to investigate the real biological mechanisms acting at the base. Furthermore, the chicken genome is now sequenced, hence the theoretical identification of all genes product by means of mass spectrometry is now possible. The approach shows however some disadvantages and limitations. The scarce automation makes this method quite laborious and time consuming. Only a part of the entire proteome is easily analyzable by means of this technique, firstly because of its variable nature, that change in response to the external stimuli, secondly because only one tissue among other has been chosen for the analysis, and finally because the fractionation of the protein sample further reduced the number of analyzable proteins. Moreover, the statistical approaches are not yet well developed and most of them are coming from different fields, such as transcriptomics (with whom it shares may important features). Lastly, there is a big interpretative gap from the identification of the most relevant expressed proteins to the explanation of the biological differences mainly because of a lack of knowledge about the function of many proteins. Hence, the detected differences in protein expression, that were here successful in measuring breed differentiation, cannot be used univocally to explain the biological factors involved in this phenomenon.

Figure 1

Ward minimum distance cluster plot for Pèpoi (pp), Padovana (pd) and Ermellinata (er) individuals



CONCLUSIONS

The results are promising. This approach can successfully integrate information obtainable with conventional methods for the study of biodiversity. Although this technique does not quantitatively measure the genetic variation within a breed or a population, at least with the tools here used, it succeeds in unfolding the relations among breeds and single individuals. In addition this technique enables to obtain information on the true differences existing at the proteome level among the breeds.

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